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ORIGINAL ARTICLE

Isolation of phytosterols of *Dalbergia ecastophyllum* (L.) Taub. (Leguminosae) and modulation of antibiotic resistance by a possible membrane effect

Gláucia M.M. Guedes [a], Rosimeire S. Albuquerque [a], Rafela S. Soares-Maciel [b], Maria A. Freitas [a], Viviane A. Silva [b], Edeltrudes O. Lima [b], Micheline A. Lima [b], Emidio V.L. Cunha [b], Henrique D.M. Coutinho [a,*]

[a] *Laboratório de Microbiologia e Biologia Molecular, Universidade Regional do Cariri, Crato (CE), Brazil*
[b] *Laboratório de Tecnologias Farmacêuticas, Universidade Federal da Paraíba, João Pessoa (PB), Brazil*

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Abstract Globally there are a larger number of strains of microorganisms resistant to multiple drugs mainly due to misuse and indiscriminate, resulting in increased morbidity, costs inherent benefits of health care, as well as mortality rates for infections. As a result of this a large number of researches have been conducted emphasizing the antimicrobial properties of plant products. In this study, the ethanol extract and hexane fraction of *Dalbergia ecastophyllum* (L.) Taub. (Leguminosae) have been used to evaluate the antibacterial and antifungal activity and for modulating the resistance of antimicrobials against bacterial strains of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* and fungal strains of *Candida krusei*, *Candida tropicalis*. The antibacterial and modulatory activity was determined by microdilution. Inhibition of the growth of bacteria and fungi tested extract was $\geqslant 1024$. The activity was enhanced when aminoglycosides were associated with sub-inhibitory concentrations of the ethanol extract and hexane fraction of *Dalbergia ecastophyllum*. Therefore, it is suggested that the ethanol extract and hexane fraction

* Corresponding author at: Departamento de Química Biológica, Universidade Regional do Cariri – URCA, Rua Cel. Antonio Luis 1161, Pimenta, 63105-000 Crato-CE, Brazil. Tel.: +55 (88) 31021212; fax: +55 (88) 31021291.
E-mail address: hdmcoutinho@gmail.com (H.D.M. Coutinho).
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of *Dalbergia ecastophyllum* (L.) Taub. (Leguminosae) can be used as a source of natural products with a view to changing the resistance of these microorganisms to antimicrobials.

1. Introduction

Improper use of some antimicrobials is causing the drugs to lose their effectiveness very quickly, which makes necessary for the development of new drugs and effective management techniques. Resistance is a phenomenon characterized not only by evolutionary pressure, but especially by the indiscriminate and irrational use of antimicrobial therapeutics (Coutinho et al., 2008).

The species *Pseudomonas aeruginosa* is responsible for a variety of infections, such as those affecting the skin, urinary tract, eye and ear. The wide distribution of *Pseudomonas* in the environment is ensured by its non-fastidious growth requirements, and *Pseudomonas* possess many structural factors, enzymes and toxins that enhance their virulence, and make them resistant to most common antibiotics (Murray et al., 2004). *Staphylococcus aureus* is often found colonizing the natural microbiota, especially the skin, and with the breakdown of skin barriers or decrease of immunity can become pathogenic. It causes a variety of infections, such as infections of the skin and subcutaneously, post-surgical infections, osteomyelitis, pneumonia, abscesses, endocarditis and bacteremia (Gelatti et al., 2009). *Escherichia coli* is a gram negative bacterium belonging to the family Enterobacteriaceae normally found in the intestine of being endothermic, becoming recognized as a pathogen and a harmless commensal versatile (Vogt, 2005).

Another problem associated with microorganisms is increased opportunistic fungal infections. Although a number of commercially available antimycotics have increased in recent years, they are still at a disadvantage when compared to antibacterial drugs. Additionally, resistance to antifungal drugs has represented a major challenge for clinical investigations (Batista et al., 1999).

The phenomena of antibiotic resistance are increasing despite the advances in the development of new drugs (Keith et al., 2005) and the combination with natural products of plants that can alter the effect of antibiotics, either increasing or decreasing the antibiotic activity can be an interesting alternative to overlap this problem (Coutinho et al., 2008).

Most of the biological activities exhibited by the medicinal plant are provided by the products of secondary metabolism (Adam et al., 1998). Through phytochemical screening of plant extracts, it is possible to identify the presence of several classes of secondary metabolites that exhibit a wide variety of biological activities such as antimicrobial, antioxidant, antitumor and anti-ophidian (Matias et al., 2010; Daferera et al., 2003).

Dalbergia ecastophyllum (L.) Taub. (Leguminosae), popularly known as rabo-de-bugio, has a traditional use of its roots and barks combating uterine inflammation and anemia. Studies show that propolis from *D. ecastophyllum* has demonstrated several biological activities (Daugsch et al., 2008). Because of that, this study was to evaluate the in vitro antimicrobial activity and modulate the ethanol extract and hexane phase of *D. ecastophyllum* against bacterial strains in order to identify this natural product as a new strategy to combat resistance of microorganisms to antimicrobials (see Fig. 1).

2. Experimental

2.1. Bacterial strains

The bacterial strains used in the MIC were: *E. coli* ATCC 10536, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 15442. To the modulation assay were used the multiresistant strains: *E. coli* 27, *S aureus* 358 and *P. aeruginosa* 22. All strains were maintained on slants with Heart Infusion Agar (HIA, Difco laboratorises Ltda.). Before testing, the cells were cultured at 37 °C for 24 h in brain heart infusion (BHI, Difco Laboratories Ltda.) (Table 1).

2.2. Plant material

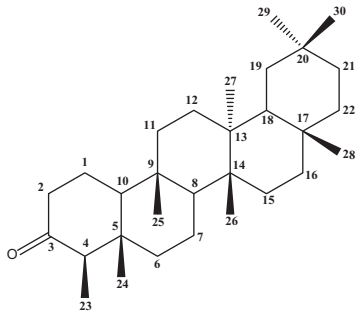
The plant *D. ecastophyllum* was investigated on how the ethanol extract and hexane fraction were obtained from its leaves, in order to evaluate the antibacterial activity of these samples facing the strains of pathogenic microorganisms. Plant species were collected near the Bessa Mangrove, Paraíba – Brazil. The plant material was identified by Evelise Locatelli, curator of the Herbarium Systematics – Federal University of Paraiba, where is deposited exsicata: JPB45738. The extract and fraction were produced by Dr. Micheline Lima, according with the methodology described by Coutinho et al. (2008).

2.3. Processing of the chromatographic hexane phase of the ethanol extract

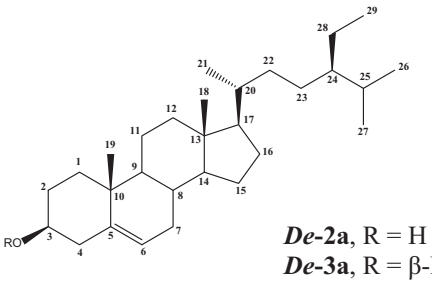
An aliquot of 15.0 g of the hexane phase (column 1.0) of crude ethanol extract of the aerial parts of *D. ecastophyllum* was subjected to column chromatography using a stationary phase silica gel and 60 were used as the mobile phase hexane, CH_2Cl_2, EtOAc and MeOH alone or in binary mixtures with increasing polarity gradient, the fractions are concentrated on a rotary evaporator pressure reduced. The fraction 55/67 (334.0 mg) was subjected to column chromatography using new adsorbent such as silica flash (Column 1.1) as eluents and hexane, CH_2Cl_2, EtOAc and MeOH alone or in binary mixtures with crescent polarity. In this column, we collected 96 fractions of 20 mL each, CCDA analyzed and assembled according to their Rf's. Fraction 13 (6.0 mg) presented in the form of white crystals, was subjected to $_1$H and $_{13}$C NMR spectroscopy encoding the substance as De-4.

2.4. Preparation of test solution

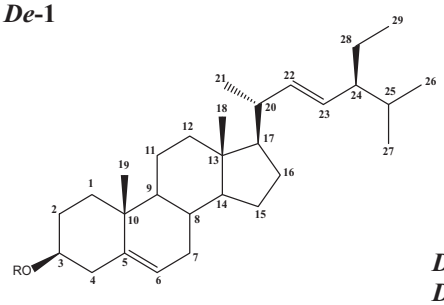
The solution used in the tests was prepared at an initial concentration of 100 mg/ml, dissolved in 1 ml DMSO, then diluted in distilled water to a concentration of 1024 μg/ml.



***De*-1**



***De*-2a**, R = H
***De*-3a**, R = β-D-Gl



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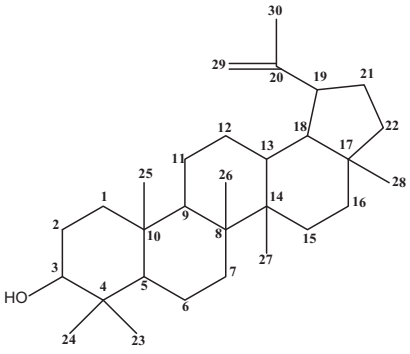
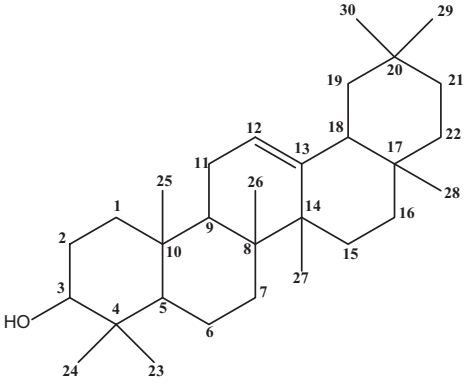
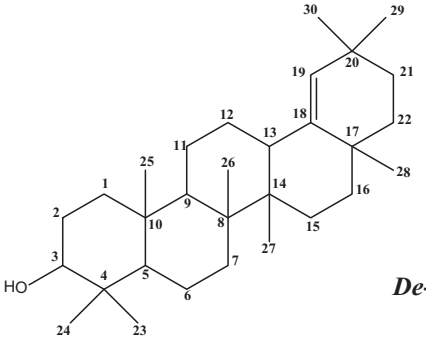
***De*-3b**, R = β-D-Gl



***De*-**



***De*-4b**



***De*-4c**

De-1 = Friedelan-3-one, (friedelin)

De-2 (a / b) = mixture of β-sitosterol (De-2a) and stigmasterol (De-2b)

De-3-(a/b) = mixture of sitosterol-3- *O*-β-D-glucopyranoside (De-3a) and Stigmasterol 3- *O*-β-D-glucopyranoside (De-3b).

De-4 (a / b / c) = mixture of lup-20 (29)-en-3-ol (lupeol), olean-12-en-3-ol (β-amyrin) and olean-18-en-3-ol (germanicol).

Figure 1 Phytocompounds identified from the hexane fraction of *Dalbergia ecastophyllum*. **De**–**1** = Friedelan-3-one, (friedelin). **De**–**2** (**a**/**b**) = mixture of β-sitosterol (**De**–**2a**) and stigmasterol (**De**–**2b**). **De**–**3**–(**a**/**b**) = mixture of sitosterol-3- O-β-D-glucopyranoside (De-3a) and Stigmasterol 3- O-β-D-glucopyranoside (De-3b). **De**–**4** (**a**/**b**/**c**) = mixture of lup-20 (29)-en-3-ol (lupeol), olean-12-en-3-ol (β-amyrin) and olean-18-en-3-ol (germanicol).

Table 1 Source of bacterial strains and resistance to antibiotics.

Bacteria	Source	Resistance profile
Escherichia coli 27	Surgical wound	Ast, Ax, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Clo, Im, Can, Szt, Tet, Tob
Escherichia coli ATCC10536	–	–
Staphylococcus aureus 358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net
Staphylococcus aureus ATCC25923	–	–
Pseudomonas Aeruginosa 22	Catheter tip	Cpm, Ctz, Im, Cip, Ptz, Lev, Mer, Ami

Ast – Aztreonan; Ax – Amoxicillin, Amp – Ampicillin; Ami – Amicilina; Amox – Amoxicillin; Ca – Cedroxil; Cfc – cefaclor; Cf – Cephalothin, Caz – Ceftazinidima; Cpm – Cefepime; Ctz – ceftazidime, Cip – Ciproflaxacin; Clo – Chloramphenicol; Im – Imipenem; Can – Kanamycin; Szt – sulphametrim; Lev – levofloxacin; Tetraciclina – Tet; Tob – Tobramicina; Oxa – Oxacillin; Gen – Gentamicin, Meropenem – MER; Neo – Neomycin; Piperacillin – Tazobactam – Ptz; paromomycin – To, But – Butirosin; Sis – sisomicin, Net – Netilmicin; (–) absence of resistance or endurance without relevance.

2.5. Drugs

The antibacterial drugs used at a concentration of 5000 μg/ml were: Amikacin, Gentamicin and Neomycin. All were prepared according to the manufacturer's instructions.

2.6. Antibacterial activity and modulation of drug

The minimum inhibitory concentration (MIC) was determined in a microdilution assay (Javadpour et al., 1996). An amount of 100 mL of each strain suspended in brain heart infusion broth (BHI) was dispensed into each well of a microdilution plate. In each cavity was deposited 100 μl of solution serial dilution of extract/fraction, with their final concentrations ranging from 1024 to 2 μg/ml. MIC was recorded as the lowest concentration that inhibited the growth. The minimum inhibitory concentration was determined for the antibiotic in the BHI broth microdilution assay using suspensions of 10^5 CFU/ml and a range of drug concentration 5000 μg–2.5 g/ml for antibacterial. According to Coutinho et al. (2008) evaluation of extracts as modulators of antimicrobial resistance, the MIC of antibiotics was determined in the presence or absence of the ethanol extract of *D. ecastophyllum* (BSE) and hexane fraction of *D. ecastophyllum* (FH), in sub-inhibitory concentrations (MIC/8) and the plates were incubated for 24 h at 37 °C.

3. Results and discussion

As shown in Table 2, the ethanol extract and hexane fraction showed no clinically relevant antibacterial activity against the assayed strains (MIC $\geqslant$ 1024). As can be seen in Table 2, the ethanol extract (EE) had success when combined with aminoglycosides against *E. coli* 27, there was a MIC lowering of 31 and 8 times when combined with gentamicin and neomycin respectively.

The mechanisms by which the extracts could inhibit the growth of microorganisms are varied and may be in part due to the hydrophobic nature of some components. These components can interact with lipid bilayer of the cell membrane and affect the respiratory chain and energy production (Nicolson et al., 1999), or even make the cell more susceptible to antibiotics, leading to disruption of vital cellular activity (Burt, 2004). Interference with the enzyme systems of bacteria can also be a potential mechanism of action (Wendakoon and Sakaguchi, 1995).

With *S. aureus* 358 in association extract antibiotic, an increase of 3 points of the Neomycin MIC, featuring an antagonism. No effect was seen when done association with hexane fraction (FH). The main mechanism of resistance to aminoglycosides in staphylococci is drug inactivation by cellular enzymes modifying aminoglycosides. Several distinct loci encoding such modifying enzymes were characterized in

Table 2 MIC demonstrating the Antibacterial activity and modulatory antibiotic effect of EEB against strains of *E. coli*, *S. aureus* and *P. aeruginosa* (μg/mL).

	E. coli 27		*S. aureus 358*		*P. aeruginosa 22*	
Extract/antibiotic	Alone	+EE	Alone	+EE	Alone	+EE
EEB	$\geqslant$1024	–	$\geqslant$1024	–	$\geqslant$1024	–
Gentamicin	312.5	9.76	4.88	4.88	2.44	19.53
Amycacin	39.06	39.06	78.12	78.12	156.25	156.25
Neomycin	156.25	19.53	19.53	78.12	78.12	78.12
Extract/antibiotic	Alone	+FH	Alone	+FH	Alone	+FH
FH	$\geqslant$1024	–	$\geqslant$1024	–	$\geqslant$1024	–
Gentamicin	312.5	2.44	4.88	4.88	19.53	2.44
Amycacin	39.06	39.06	78.12	78.12	156.25	19.53
Neomycin	156.25	19.53	19.53	19.53	78.12	78.12

EE – ethanol extract of *Dalbergia ecastophyllum;* FH – hexane fraction of *Dalbergia ecastophyllum.*

staphylococci. Clinically, the most important of these region coding for enzyme activities acetyltransferase (AAC), adenylyltransferase (ANT) and phosphotransferase (APH). Aminoglycosides modified into amino groups by the enzyme AAC or hydroxyl groups or by enzyme ANT APH, lose the ability to bind to ribosomes and therefore not further inhibit protein synthesis of the bacterial cells (Paulsen et al., 1997). When was done the phytochemical screening of the hexane fraction, was identified some classes of secondary metabolites present in this fraction: friedelin as terpenes and mixture of lupeol, b-amyrin and germanicol and steroids as a mixture of b-sitosterol and stigmasterol and B-sitosterol and stigmasterol glycosylated. According Bresciani et al. (2000) and Carvalho and Carvalho, (2001), nonpolar solvents such as ether and hexane allow the extraction of groups steroids, coumarin esters, sesquiterpene lactones and terpenoids.

Table 2 shows the action of the hexane fraction (FH), with *Ec* 27 the MIC of Neomycin was lowered 8 times and the Gentamicin 128 times. In the case of Pa-22, the fraction combined with amikacin and gentamicin enhanced 8 times the antibiotic activity of these drugs. This favorable result for gram negative modulation can be justified because these bacteria present a higher fat content (Vargas et al., 2004). This allows a greater affinity with the components identified in this fraction. The mechanism of action of antimicrobial activity of terpenes is not fully understood but it is speculated that involves the disruption of the cell membrane by lipophilic compounds (Cowan, 1999). This break allows for greater penetration of the antibiotic.

Terpenes or terpenoids are active against bacteria (Ahamd et al., 1993). The isolation and identification of various terpenes with antinociceptive effect, anti-inflammatory, antimicrobial, antiparasitic, trypanocidal, larvicide, have been demonstrated in preliminary studies (Sartori, 2005).

Studies involving this species has been reported, according to Daugsch et al. (2008) *D. ecastophyllum* is the botanical origin of propolis (propolis group 13) and has antimicrobial activity, and the ethanol extracts of the propolis group 13 showed antibacterial activity against *S. aureus*, *Salmonella typhimurium, Streptococcus mutans*. Results The results show a broad spectrum against various microorganisms (fungi, bacteria, viruses, protozoa). Although important biological antioxidant properties, and immunomodulatory and cytotoxic activities has been proven (Bankova et al., 2000). Silva (2008) showed that both the ethanol extracts of propolis as the ethanol extracts of *D. ecastophyllum* resin exhibited high antimicrobial activity.

The verified data point extract and fraction of *D. ecastophyllum* were used as a potent modulator of resistance to bacteria, since they reduced the MIC of the antibiotic assayed. Research is to be developed further that can support in vivo testing and therapeutic use in future. According with the results, these natural products can be an alternative against the rise of bacterial drugs resistance, with the positive point that, by the use of lower concentrations of natural products and drugs, the risk of toxicity is lower.

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